UNIVERSITI PUTRA MALAYSIA

CLONING OF CHICKEN ANAEMIA VIRUS (CUX-I) VPI GENE INTO A *LACTOCOCCUS* EXPRESSION VECTOR pMG36e

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CLONING OF CHICKEN ANAEMIA VIRUS (CUX-1) VP1 GENE INTO A *LACTOCOCCUS* EXPRESSION VECTOR pMG36e

By

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CLONING OF CHICKEN ANAEMIA VIRUS (CUX-1) VP1 GENE INTO A *LACTOCOCCUS* EXPRESSION VECTOR pMG36e

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Lactococcus lactis is a non-pathogenic and non-colonising bacterium, which is being developed as a vaccine delivery vehicle for immunisation by mucosal routes. To determine whether lactococci can express the Chicken Anaemia Virus capsid protein (VP1) in immunogenic form, we have constructed a recombinant DNA, which constitutively expresses the VP1 as N-terminal fusion protein with Open Reading Frame-32 under the control of lactococci promoter P32 in vector pMG36e.

Chicken anaemia virus (CAV) is a member of the newly categorised *Circoviridae*, a family of circular negative single-stranded DNA viruses. CAV VP1 gene (1.4 kb) was amplified by PCR from CAV genome which was isolated from infected Marek's disease virus transformed lymphoblastoid cell line (MDCC-MSB1) cell lysate and cloned into pCR®2.1-TOPO vector for sequencing. The sequenced VP1 gene, lacking its own promoter, was cloned into the lactococcal expression vector

pMG36e and transformed into *E. coli* JM109 as the intermediate host. The recombinant plasmid was sub-cloned into *L. lactis* MG1363 by electroporation. Several independent verifying methods such as sequencing, Southern hybridisation and restriction enzymes analysis have confirmed the insertion of VP1 gene into pMG36e. The complete sequence of VP1 gene has 99.65% homology compared to the published CAV Cux-1 VP1 gene. A total of 5 base difference was seen to result in change of 3 amino acids sequence in the polypeptide chain. The expression of fusion protein was studied until transcriptional level. Transcription of fusion gene from strains of recombinant *L. lactis* MG1363 were analysed by Northern hybridisation. Northern hybridisation has detected transcription of fusion gene in recombinant *L. lactis* MG1363 carrying pMG36e-VP1and produced mRNA with the size of 1.4 kb.

On the basis of these results, it is concluded that the recombinant DNA pMG36e was successfully constructed in bacteria *E. coli* JM109 and subcloned into *L. lactis* MG1363. The recombinant DNA in *L. lactis* MG1363 is capable to express the fusion protein up to transcriptional level. Therefore, recombinant *L. lactis* MG1363 can be used as a potential vaccine delivery system against CAV infections.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENGKLONAN GEN VP1 VIRUS ANAEMIA AYAM (CUX-1) KE DALAM SATU VEKTOR PENGEKPRES *LACTOCOCCUS* pMG36e

Oleh

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Lactococcus lactis ialah sejenis bacteria yang bukan patogenik dan tidak mengkoloni yang telah direka sebagai agen untuk pengangkutan vaksin untuk tujuan immunisasi melalui kaedah mucosal. DNA rekombinan yang mengekspres VP1 sebagai terminal N protein fusion dengan Open Reading Frame-32 di bawah pengawalan promoter P32 di dalam vektor pMG36e telah dibina untuk menentukan sama ada lactococci boleh mengekspres protein kapsid (VP1) yang immunogenik.

Virus anemia ayam (CAV) adalah ahli kepada keluarga baru yang dinamakan *Circoviridae*, iaitu kumpulan yang mempunyai DNA bulatan negatif satu rantai. Gen VP1 (1.4 kb) telah diamplifikasikan melalui PCR daripada genom CAV yang diekstrak dari sel MDCC-MSB1 yang telah dijangkiti oleh CAV dan diklonkan ke dalam vektor pCR®2.1-TOPO untuk penentuan jujukan DNA. Gen VP1 tanpa

promoter telah dimasukkan ke dalam vektor pengekpres pMG36e dan ditransfom ke dalam *E. coli* JM109 sebagai perumah perantara. DNA recombinan ini kemudian dipindahkan ke dalam bakteria *L. lactis* MG1363 dengan kaedah electroporation. Beberapa kaedah pengesahan termasuk penentuan jujukan DNA, analisis dengan enzim pembatas dan penghibridan Southern telah menunjukkan bahawa gen VP1 telah berjaya dimasukkan ke dalam vektor pMG36e. Keseluruhan penentuan jujukan DNA menunjukkan gen VP1 mempunyai homologi 99.65% berbanding dengan jujukan DNA gen VP1 CAV Cux-1 yang telah diterbit. Sejumlah 5 perbezaan bes dalam jujukan DNA telah membawa perubahan kepada 3 jujukan asid amino di dalam rantai polipeptida. Kajian terhadap pengekspresan protein fusion telah mencapai ke tahap transkripsi. Transkripsi gen fusion daripada strain *L. lactis* MG1363 rekombinan telah dianalisis dengan penghibridan Northern. Penghibridan Northern menunjukkan bahawa transkripsi gen fusion telah belaku di dalam strain *L. lactis* MG1363 yang mengandungi pMG36e-VP1 dan menghasilkan mRNA bersaiz 1.4 kb.

Berdasarkan hasil kajian yang diperolehi, ia boleh disimpulkan bahawa DNA rekombinan telah berjaya dibina dalam bakteria *E. coli* JM109 and disubklon ke dalam *L. lactis* MG1363. DNA rekombinan di dalam *L. lactis* MG1363 berupaya mengekspres protein fusion sehingga tahap transkripsi. Oleh itu, *L. lactis* MG1363 rekombinan berpotensi untuk digunakan sebagai sistem pengangkutan vaksin bagi mencegah jangkitan CAV.

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I certify that an Examination Committee met on 19th August, 1999, to conduct the final examination of Ng Perk Tsong, on his Master of Science thesis entitled "Cloning Of Chicken Anaemia Virus (Cux-1) VP1 Gene into A *Lactococcus* Expression Vector pMG36e" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Signed

Candidate.
Ng Perk Tsong
21st February 2000

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AP	-	alkaline phosphatase
ATF	-	Adenovirus transcription factor
bp	-	base pair
BSA	-	bovine serum albumin
CAV	-	chicken anaemia virus
CCC	-	covalently closed circular
Cm^r	-	chloramphenicol resistant
d/ddNTP	-	de/dideoxynucleotidetriphosphate
DEPC	-	diethyl pyrocarbonate
DIG	-	digoxigenin
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediamine tetraacetate
ELISA	-	enzyme-link immunosorbant assay
Em	-	erythromycin
Em^r	-	erythromycin resistant
EtBr	-	ethidium bromide
GST	-	glutathione-S-transferase
HMW	-	high molecular weight
kb	-	kilo base pair
kDa	-	kilo Daltons
LB	-	Luria Bertani
M	-	molarity
MCS	-	multiple cloning site

MDCC-MSB1	-	Marek's disease virus transformed lymphoblastoid cell line
Mol	-	mole
OD	-	optical density
ORF	-	open reading frame
PCR	-	polymerase chain reaction
RBS	-	ribosomal binding site
RE	-	restriction enzyme
RF	-	replicative form
RNA	-	ribonucleic acid
rRNA	-	ribosomal RNA
mRNA	-	messenger RNA
rpm	-	revolution per minute
SDS-PAGE	-	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSI	-	single-stranded-intermediate
SS	-	single-stranded
STE	-	sodium tris-EDTA
TAE	-	tris-acatate-EDTA
TBE	-	tris-borate-EDTA
TTFC	-	tetanus toxin fragment C
X-gal	-	5-bromo4-chloro-3-indolyl-β-D-galactopyranosidase

CHAPTER I

INTRODUCTION

Chicken anaemia virus (CAV) is a member of the newly categorised family *Circoviridae*. CAV is a small icosahedral shaped DNA virus. The virus causes a syndrome in chicken characterised by aplastic anaemia, lymphoid depletion, subcutaneous and intramuscular haemorrhages and increased mortality (Yuasa *et al.*, 1979). It also causes the destruction of the erythroblastoid cells in bone marrow and depletion of cortical $CD4^+$ lymphocytes in the thymus, resulting in acute anaemia and immunodeficiency (Jeurissen *et al.*, 1989).

Current vaccines for CAV are based on either live attenuated or inactivated CAV (Steenhuisen *et al.*, 1994); however, there are drawbacks in each approach. The use of attenuated viral vaccines carries the risk that the virus is not truly attenuated. Consequently, the chickens may become infected by CAV. Such infections are often asymptomatic and result in lower growth and weight gain rates.

Several researchers have studied cloning and sequencing of genomic DNA from CAV (Claessens *et al.*, 1991; Noteborn *et al.*, 1991; Meehan *et al.*, 1992). Gene sequences have been isolated that could be used to make a subunit vaccine against CAV. Amplified CAV genes were cloned into plasmids and transformed to suitable host such as bacteria and baculovirus (Pallister, 1994; Kato *et al.*, 1995; Koch *et al.*, 1995). The expression of the cloned gene(s) was determined by screening of the fusion protein. Since subunit vaccines utilise recombinantly expressed proteins of the virus and not the virus itself, the problem of infections inherent in the use of attenuated viruses as vaccines can be overcomed. Furthermore, manufacture of recombinant proteins for use as subunit vaccines are less expensive than the culture and inactivation of the viruses used in inactivated viral vaccines.

Lactococcus is one of the important groups of lactic acid bacteria. It is a Gram-positive cocci and produces lactic acid during fermentation process. *Lactococcus* consists of two major subspecies: *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. This bacterium has importance for major economic activities, primarily their use in a variety of dairy fermentation such as cheese production. Much effort has been put into the development of methods to modify these microorganisms genetically. Transformation systems were developed and special-purpose cloning vectors became available (Kok , 1991).

Increasing knowledge of the molecular biology of the lactic acid bacteria creates the interest in the development of alternative bacterial vaccine vectors based on

the harmless and non-pathogenic bacteria as cloning and antigen vehicles. Such a system would be particularly suited for mucosal immunisation of geriatric and paediatric populations. In contrast to conventional vaccines, which are largely derived from attenuated pathogens such as *Salmonella* and BCG (Dougan *et al.*, 1993; Stover *et al.*, 1993), the lactic acid bacteria are generally regarded as safe organisms making them particularly attractive for use as antigen delivery vehicles. *L. lactis* was able to produce high levels of heterogeneous antigen expression *in vitro* prior to inoculation of the bacteria (Norton *et al.*, 1995). Therefore this system does not require *in vivo* expression of the antigen in the host. Furthermore *L. lactis* has a low innate immunogenicity and it is possible to carry out sequential oral inoculations of this bacterium without inducing a significant increase in the antibacterial antibody response, thus reducing the risk of interference from antibacterial antibodies (Norton *et al.*, 1995).

Objectives

The objective of this study is to clone the VP1 gene of the CAV Cux-1 strain into the lactococcal expression vector pMG36e. The VP1 gene is first amplified by the polymerase chain reaction and then cloned into the pCR®2.1 cloning vector for product verification. The VP1 gene is then subcloned in-frame with ORF-32 into pMG36e and transformed into *L. lactis* MG1363. The fusion gene expression in *L. lactis* MG1363 is examined using Northern hybridisation.

CHAPTER II

LITERATURE REVIEW

Chicken Anaemia Virus (CAV)

Introduction

CAV is a member of the newly categorised *Circoviridae*, a family of circular negative single-stranded DNA viruses that also includes the porcine circovirus and the beak and feather disease virus of parrots (Studdert, 1993). These viruses are grouped together on the basis of a common genome form, but there were no similarity in amino acid composition, open reading frame (ORF) arrangement, nor transcriptional machinery have been identified. The virion is a small, icosahedral DNA virus with an average diameter of 25 nm (Goryo *et al.*, 1987). It is resistant to treatments with chloroform, ether, pH 3, heating at 80°C for 5 min and can be cultured in Marek's disease virus transformed chicken lymphoblastoid cell line (MDCC-MSB1).

CAV was first isolated from chicken in Japan by Yuasa *et al.* (1979). Since then it has been detected and isolated in other part of the world such as Germany (von Bülow *et al.*, 1983; Vielitz and Landgraf, 1988), Sweden (Engstrom, 1988), United Kingdom (Chettle *et al.*, 1989; McNulty *et al.*, 1989), the USA (Goodwin *et al.*, 1989; McNulty *et al.*, 1989; Rosenberger and Cloud, 1989; Lucio *et al.*, 1990), Australia (Firth and Imai, 1990), and China (Zhou *et al.*, 1997). However, only one serotype of CAV has been found, so far and these were all pathogenic for young chickens.

In Malaysia, preliminary study to determine the presence of CAV in flocks of commercial chicken was done by Rozanah *et al.* (1995). A total of 322 serum samples were randomly collected from layers, broilers and parent stock of different ages from 13 farms in 3 states of Malaysia (Selangor, Negeri Sembilan and Johor) and tested with enzyme-link immunosorbant assay (ELISA) kit using CAV hyperimmune antiserum. Liver and spleen of CAV antibody positive chicken were further tested for the presence of CAV antigen by indirect immunofluorescent and immunoperoxidase staining. The results showed that CAV antibody could be detected in 218 (61.4%) sera out of 355 sera tested. The antibodies were detected in all age groups of chicken from one day old to 70 weeks old including layers, broiler and parent stock flocks. Antigen of CAV was detected in the nuclei and cytoplasm of hepatocytes from 2 antibody positive chickens. The nuclei of the hepatocytes were stained intensely while the cytoplasm was lightly stained. This finding confirmed that Malaysian chicken flocks are not free from CAV and the infection is highly prevalent.

Genomic Organisation and Gene Products of CAV (Cux-1)

The CAV Cux-1 strain genome was first reported to be composed of a single–stranded circular DNA molecule of 2319 nucleotides (Noteborn *et al.*, 1991). However, the genome size of most naturally occurring isolates including Cux-1 was reported to be 2298 bases because of the lack of one copy unit of the 21-base tandem repeat located on the non-coding region on the genome (Claessens *et al.*, 1991; Meehan *et al.*, 1992).

A study on CAV transcription by Noteborn *et al.* (1992) showed that the major transcript from the CAV genome is an unspliced messenger (mRNA) of about 2100 nucleotides. Its transcription start point and poly(A)-addition site are located at nucleotides 354 and 2317 of the CAV sequence, respectively. *In vitro* translation experiments provide evidence that the major CAV open reading frame encodes a 52 kDa protein by using the fifth AUG as a start codon of the unspliced CAV mRNA. This 2.0 kb transcription product was further confirmed by Phenix *et al.* (1994) by using strand-specific riboprobes representative of either strand of the chicken anaemia virus replicative form (RF) DNA. The results indicated that only one strand of the RF was transcribed to produce a major 2.0 kb transcript and it shown that the encapsidated DNA was of negative sense, which was not transcribed. Primer extension analysis located a single transcriptional start site at nucleotide position 360 bp (Figure 1) of the CAV sequence (Phenix *et al.* 1994). Northern blot analysis using a series of genomic probes indicated that the 2.0 kb transcript was devoid of splicing

and identified a non-transcribed region of the genome. Both studies showed that only plus strand of CAV DNA transcribed and VP1 gene transcription started at the firth AUG of the unspliced mRNA as start codon.

The non-transcribed region of the CAV DNA located upstream of the three major ORFs (position 2207-385 bp, Figure 1)) encompasses a region high in GC content, a poly(A) signal and four copies of an 18 repeat element. The non-transcribed region was shown to possess promoter activity, enhancing the expression of human growth hormone reporter gene in a transient gene expression assay (Phenix *et al.* 1994). This region contains sequences considered to have roles in the initiation and regulation of transcription (Claessens *et al.*, 1991; Noteborn *et al.*, 1991; Meehan *et al.*, 1992). The promoter region, 'TATA box' (position 329-336) is believed to be the RNA polymerase II transcription factor binding sequence element. The 'TATA box' is found 50 nucleotides upstream of the initiation codon of the VP2 ORF. The promoter enhancer region of CAV contains four direct repeats, which are unique for CAV DNA. Each repeat contains a sequence that was homologous to the one that binds the adenovirus transcription factor (ATF) site. Lee *at al.* (1987) reported that some ATF sites are activated by the E1A protein of adenovirus. Moreover, in dual infection of chickens, adenovirus is enhanced by CAV, as is shown by higher virus titres (von Bülow *et al.*, 1986).

The CAV Cux-1 2319 bp DNA (Figure 1) contained all the genetic information needed for the complete replication cycle of CAV. Noteborn *et al.* (1991)